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Bacteria detection based on the evolution of enzyme-generated volatile organic compounds: Determination of *Listeria monocytogenes* in milk samples

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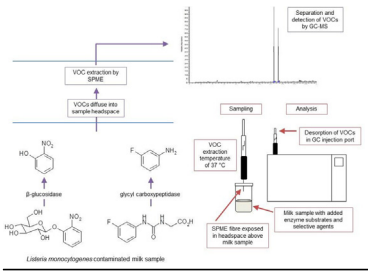
HIGHLIGHTS

- Rapid detection of *Listeria monocytogenes* contamination in food.
- Use of VOC liberating enzyme substrates.
- Analysis of VOCs by HS-SPME GC–MS.
- Use of selective agents to aid detection.

GRAPHICAL ABSTRACT



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ABSTRACT

The rapid detection of *Listeria monocytogenes* contamination in food is essential to prevent food-borne illness in humans. The aim of this study was to differentiate non-contaminated milk from milk contaminated with *L. monocytogenes* using enzyme substrates coupled with the analysis of volatile organic compounds (VOCs). The method is based on the activity of β-glucosidase and hippuricase enzymes and the detection of a specific VOC i.e. 2-nitrophenol and 3-fluoroaniline, respectively. VOCs were extracted, separated and detected by headspace-solid phase microextraction coupled to gas chromatography–mass spectrometry (HS-SPME GC–MS). This approach required the inclusion of the selective agent's cycloheximide, nalidixic acid and acriflavine HCl in the growth medium to inhibit interfering bacteria. The VOCs were liberated by *L. monocytogenes* provided that samples contained at least 1–1.5 × 10^2 CFU ml^{-1} of milk prior to overnight incubation. This approach shows potential for future development as a rapid method for the detection of *L. monocytogenes* contaminated milk.

1. Introduction

Listeria monocytogenes is a Gram-positive rod belonging to the genus *Listeria*, which also consists of nine further species: *Listeria ivanovii*, *Listeria welshimeri*, *Listeria seeligeri*, *Listeria grayi*, *Listeria innocua*, *Listeria marthii*, *Listeria rocourtiae*, *Listeria weihenstephanensis* and *Listeria fleischmannii*. *L. monocytogenes* causes food-borne infections in humans; symptoms of Listeriosis include gastroenteritis, blood poisoning and meningitis. All foods can potentially become contaminated with *L. monocytogenes*; foods most commonly associated with outbreaks include cooked meats, pâtés, shellfish and dairy products [1]. *L. monocytogenes* can survive and grow in extreme environmental conditions. It is able to grow over a temperature range of <0–45 °C which includes refrigeration temperatures, as well as extreme pH (for example, in excess of pH 9) and high salt concentrations [2]. However, identifying the source of *L. monocytogenes* contamination is often difficult. The ability of *L. monocytogenes* to survive under adverse conditions means that low levels of strains can persist in the environment for long periods of time [3]; for example, there is a

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risk that *L. monocytogenes* can proliferate if food products are refrigerated for a lengthy time period. In addition food samples are not always available to test for contamination due to the long incubation period often required for symptoms of food poisoning to develop [4]. Identifying the source of contamination is important to enable the rapid removal of foods from food suppliers and distributors, hence reducing the size of any outbreak or potential outbreak of Listeriosis [1].

The amount of *L. monocytogenes* detected in contaminated food samples varies considerably; enrichment methods are used to enable *L. monocytogenes* to grow to detectable levels. Enrichment broths incorporating selective agents such as nalidixic acid and acriflavin to inhibit background flora are used to isolate *L. monocytogenes* from contaminated foods. *L. monocytogenes* identification methods include culturing, biochemical tests and immunological assays [5]. These procedures are often time-consuming as sufficient bacterial growth is required for identification purposes. A rapid, high throughput method for the analysis of food samples that is able to detect *L. monocytogenes* contaminated foods would be highly desirable.

The hippuricase test can be one of several biochemical tests employed to identify *L. monocytogenes*. Hippuricase (glycyl carboxypeptidase) hydrolyses the peptide link in hippuric acid [6] and once hippuric acid has been hydrolysed into benzoic acid and glycine, glycine is detected by the development of a purple colour after the addition of ninhydrin [7]. *L. monocytogenes*, as well as other *Listeria* species, give positive results for the hippuricase test [8]. In addition, a VOC generating substrate targeting β-glucosidase used in conjunction with a glycyl carboxypeptidase substrate could form the basis of a simple and rapid method for the detection of *L. monocytogenes* contaminated food. The concept of using an enzyme substrate that liberates a specific VOC in the presence of bacteria has previously been reported [9–11]. In 1991 Snyder and co-workers published two papers [9,10] investigating the use of the enzyme substrate (2-nitrophenyl-β-D-galactopyranoside) in the presence of *Escherichia coli* in pure cultures to liberate the VOC, 2-nitrophenol with detection via a hand-held ion mobility spectrometer. This work, using an ion mobility spectrometer and enzyme substrates, was extended by others [11] and applied to bacteria of a food safety concern, specifically *E. coli* (using the substrate 2-nitrophenyl-β-D-glucuronide), *Aeromonas* spp. (using the substrate 2-nitrophenyl-β-D-galactoside), *Listeria* spp. (using the substrate 2-nitrophenyl-β-D-glucopyranoside) and *Staphylococcus aureus* (using the substrate 2-nitrophenyl-β-D-galactoside-6-phosphate) each liberating the VOC 2-nitrophenol. Alternative approaches for the detection of the emitted VOC from enzyme substrates are also being developed [12–16]. For example, Guillemot et al. (2013) have reported the detection of 4-nitrophenol liberated by *E. coli* in the presence of the substrate 4-nitrophenyl-β-D-glucuronide and detection by colorimetric analysis [12]. Whilst other approaches have investigated the detection of VOCs, using colorimetric analysis, emitted as a result of the metabolic growth of bacteria, and without the addition of enzyme substrates [13–16].

This study utilizes the inherent presence of bacterial enzymes to provide a rapid, non-invasive approach for the detection of listeria in food samples. The addition of specific enzyme substrates, one commercially available (2-nitrophenyl-β-D-glucoside) and the other specifically designed and synthesized for this study (2-[(3-fluorophenyl) carbamoylamino]acetic acid), to liberate unique, identifiable and quantifiable VOCs i.e. 2-nitrophenol and 3-fluoroaniline. The approach has been used for the identification of *L. monocytogenes* contaminated food samples. *L. monocytogenes* has been implicated in food-borne illness originating from various food types, but this work will focus on milk samples artificially contaminated with *L. monocytogenes*.

2. Materials and methods

2.1. Chemicals/reagents

2-Nitrophenol (98%) was purchased from Sigma–Aldrich (Poole, UK) and 2-nitrophenyl-β-D-glucoside was obtained from Apollo Scientific (Stockport, UK). 3-Fluoroaniline (99%) was obtained from Alfa Aesar (Morecambe, UK).

Columbia blood agar with 5% defibrinated horse blood, brain heart infusion broth, Listeria enrichment broth and Listeria selective enrichment supplement containing cycloheximide 25.0 mg, nalidixic acid 20.0 mg and acriflavine HCl 7.5 mg 500 ml^{-1} broth were purchased from Oxoid (Basingstoke, UK).

Types of milk used: whole milk (3.6% fat), semi-skimmed milk (1.8% fat), unhomogenised milk (5.2% fat), soya milk, whole goat's milk (3.6% fat), skimmed milk (0.1% fat) and unpasteurised milk.

2.2. Instruments

The GC–MS instrument used to separate and detect bacterial VOCs was a Trace GC Ultra fitted with a Polaris Q ion trap mass spectrometer with Xcaliber 1.4 SR1 software. Separation of VOCs was carried out using a 30 m × 0.25 mm ID × 0.25 μm VF-waxMS capillary column. The S/SL injector was set at 230 °C and operated in split mode with a flow rate of 10 ml min^{-1} and a split ratio of 10. The temperature program used was: 50 °C held for 2 min then increased at a rate of 10–220 °C min^{-1} with a final 2 min hold. The carrier gas was helium with a flow rate of 1.0 ml min^{-1}. Electron ionisation (EI) was used (70 eV), and the mass spectrometer was operated in total scan mode over an m/z range of 50–650 amu. The transfer line was held at 250 °C while the ion source temperature was maintained at 260 °C.

2.3. Procedure: SPME parameters

VOCs were extracted for 10 min at 37 °C (no stirring) prior to a desorption for 2 min at 230 °C in the injection port of the GC–MS prior to separation and identification. All fibres were conditioned in the GC injection port prior to use as directed by manufacturers guidelines. All fibres were used with a manual holder.

2.4. Procedure: microbiology

All bacteria were obtained from the National Collection of Type Cultures (NCTC) at the Microbiology Department, Freeman Hospital, Newcastle upon Tyne. Table 1 lists all strains used. Bacteria were stored and subcultured weekly on Columbia blood agar with 5% defibrinated horse blood.

Table 1
List of bacteria.

Listeria species	Gram-positive bacteria	Gram-negative bacteria
Listeria monoctyogenes NCTC 11994	*Corynebacterium striatum* NCTC 764	*Escherichia coli* NCTC 12079
Listeria monocytogenes NCTC 10357	*Corynebacterium xerosis* NCTC 9755	*Salmonella enteritidis* NCTC 6676
Listeria welshimeri NCTC 11857	*Lactococcus lactis* NCTC 662	
Listeria seeligeri NCTC 11856	*Lactobacillus acidophilus* NCTC 4504	
Listeria innocua NCTC 11288	*Bacillus licheniformis* NCTC 10341	
Listeria ivanovii NCTC 11846	*Bacillus subtilis* NCTC 3610	
Listeria grayi NCTC 10815	*Bacillus cereus* NCTC 7464	
	Enterococcus faecalis NCTC 775	
	Enterococcus faecium NCTC 7171	

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2.5. Procedure for synthesis of 2-[(3-fluorophenyl) carbamoylamino] acetic acid

To glycine ethyl ester hydrochloride (1.4 g, 0.010 mol) in dichloromethane (20 ml) was added triethylamine (1.6 ml, 0.012 mol). 3-Fluorophenylisocyanate (1.2 g, 0.009 mol) was then added over 5 min. The mixture was stirred overnight and dilute hydrochloric acid (10 ml) was added. The organic layer was separated and washed sequentially with dilute hydrochloric acid (10 ml) and water (10 ml). The organic layer was dried ($MgSO_4$) and evaporated yielding a yellow oil (1.35 g). This was stirred with hot 1 M sodium hydroxide solution (20 ml) for 1.5 h and then filtered while hot. The filtrate was allowed to cool to room temperature and acidified by the addition of 2 M hydrochloric acid solution. A white solid, 2-[(3-fluorophenyl) carbamoylamino]acetic acid, was produced (0.73 g, 40%).

The analytical data for 2-[(3-fluorophenyl) carbamoylamino] acetic acid are as follows. ^{1}H NMR (d_6-DMSO, 400 MHz) δ 9.05 (1H, s, N*H*), 7.41 (1H, dt, J = 12, 2.4 Hz, Ar*H*), 7.20 (1H, m, Ar*H*), 7.00 (1H, m, Ar*H*), 6.66 (1H, m, Ar*H*), 6.42 (1H, t, J = 5.7 Hz, N*H*), 3.73 (2H, d, J = 5.7 Hz, CH_2); ^{13}C NMR (d_6-DMSO, 100 MHz) δ 173.6, 162.9 (d, J = 239 Hz), 155.5, 142.6 (d, J = 11 Hz), 130.6 (d, J = 5 Hz), 113.8 (d, J = 2 Hz), 108.0 (d, J = 21 Hz), 104.8 (d, J = 27 Hz), 41.8; HRMS (ES) calculated for $C_9H_{10}N_2O_3$ (M + H) 213.0670, found 213.0669.

2.6. Growth of bacteria and sample preparation

All bacteria were subcultured overnight at 37 °C on Columbia blood agar one day prior to preparation for VOC analysis. After overnight incubation on blood agar at 37 °C bacteria were inoculated in sterile *Listeria* enrichment broth (LEB) with added *Listeria* selective enrichment supplement. The enzyme substrates 2-nitrophenyl-β-D-glucoside and 2-[(3-fluorophenyl) carbamoylamino]acetic acid (Fig. 1) were separately dissolved in LEB and added to sterile LEB with selective agents.

Bacterial samples were prepared by measuring the absorbance of the incubated bacterial suspension at $OD_{600\,nm}$. At an absorbance reading of 0.132 (equivalent to 0.5 McFarland units) an aliquot of 100 μL of bacterial suspension (1–1.5 × 10^7 CFU) was added to a 20 ml clear vial containing 9 ml LEB with 50 μg ml^{-1} cycloheximide, 40 μg ml^{-1} nalidixic acid, 15 μg ml^{-1} acriflavine HCl, 100 μg ml^{-1} 2-nitrophenyl-β-D-glucoside and 20 μg ml^{-1} 2-[(3-fluorophenyl) carbamoylamino]acetic acid. Then, 0.9 ml whole cow's milk was added to the inoculated LEB. Preparation of all bacterial samples followed this procedure. All samples were incubated overnight at 37 °C and then subjected to volatile profiling via HS-SPME GC–MS. Uninoculated LEB with selective agents, whole cow's milk plus added substrates was tested every day of sampling.

2.7. Sample analysis using HS-SPME-GC–MS

Bacterial VOCs were extracted and concentrated using an 85 μm polyacrylate (PA) SPME fibre. A previous study [17] investigated the influence of SPME fibre type for the detection of VOCs by bacteria. It was found that the choice of SPME fibre type could influence the detection of VOCs. The PA SPME fibre was selected for this study based on those findings. In addition, the study [17] investigated the evolution of natural occurring VOCs and their variation from the same bacteria in the presence of different cultural media. The issues highlighted in that approach [17] led to the development of this new and alternate methodology using enzyme-substrates to liberate VOCs. Bacterial VOCs were separated and detected using GC–MS. VOCs were identified by both their retention times and mass spectra with confirmation of VOCs achieved using known standards and a customised mass spectral library. The VOCs 2-nitrophenol and 3-fluoroaniline were quantified using external calibration. Calibration graphs were prepared by spiking VOC standards into blank culture medium with 0.9 ml whole cow's milk. Limits of detection (LOD) and limits of quantification (LOQ) were determined as the peak area of three times the signal to noise ratio and 10 times the signal to noise ratio, respectively. Samples were considered to be negative for VOCs if VOC signal size was below that of the LOD and VOCs were also designated as absent when present at trace but not quantifiable levels, i.e. if signal size was above LOD but less than LOQ.

VOC profiles analysed and subject to statistical analysis, specifically cluster analysis, which was carried out using the nearest neighbour method and Squared Euclidean distance as an interval measurement on VOC concentration data. 2-Nitrophenol and 3-fluoroaniline concentrations (μg ml^{-1}) liberated by all three replicates per strain were used separately in calculations; any species that did not produce VOCs were excluded from analysis. Dendrograms were generated to illustrate the clustering of strains. Statistical analysis was performed on SPSS version 19 (IBM, New York, USA).

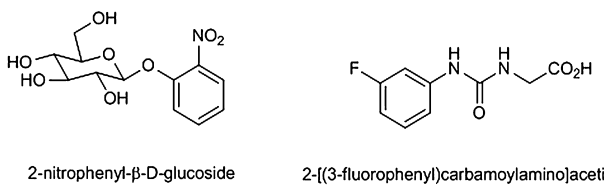

Fig. 1. Structures of 2-nitrophenyl-β-D-glucoside and 2-[(3-fluorophenyl) carbamoylamino]acetic acid.

3. Results and discussion

The selection of 2-nitrophenol and 3-fluoraniline as the emitted VOCs was done for a number of reasons. The primary reason for the selection of each VOC was their inherent unlikeliest occurrence in a natural microbiological system. By an evaluation of the scientific literature it was determined unlikely that a fluorine-containing VOC would be naturally occurring in this microbiological system; similarly, it was also considered highly improbable that 2-nitrophenol would naturally occur in this microbiological system. In additional preliminary experimental analysis also confirmed that both 2-nitrophenol and 3-fluoroaniline were volatile and could be analysed by headspace SPME-GC–MS. The VOCs 2-nitrophenol and 3-fluoroaniline were separated and identified by both mass spectra and retention times. 2-Nitrophenol and 3-fluoroaniline displayed linearity over a six point concentration range of 0.5–100 μg ml^{-1} and 0.25–20 μg ml^{-1}, respectively, with correlation coefficients exceeding 0.99 for both VOCs.

The substrates 2-[(3-fluorophenyl) carbamoylamino]acetic acid and 2-nitrophenyl-β-D-glucoside were tested with ten samples of *L. monocytogenes* NCTC 11994 inoculated in whole cow's milk with LEB plus selective agents. The VOCs 2-nitrophenol and 3-fluoroaniline were produced by 100% of inoculated whole milk samples with mean amounts of 2-nitrophenol and 3-fluoroaniline produced ± one standard deviation being 1.6 ± 0.9 μg ml^{-1} and 3.7 ± 1.3 μg ml^{-1}, respectively. VOCs were not liberated by uninoculated whole milk samples (n = 10). Fig. 2 shows the VOC profile liberated by *L. monocytogenes* with the addition of both substrates to the culture medium.

The sensitivity of the method was assessed in terms of initial inoculum size. The initial inocula prepared were: 1–1.5 × 10^7, 1–1.5 × 10^6, 1–1.5 × 10^5, 1–1.5 × 10^4, 1–1.5 × 10^3 1–1.5 × 10^2, 1–1.5 × 10^1 and 1–1.5 × 10^0 CFU ml^{-1} milk. An initial inoculum of 1–1.5 × 10^1 CFU ml^{-1} milk and 1–1.5 × 10^2 CFU ml^{-1} milk was

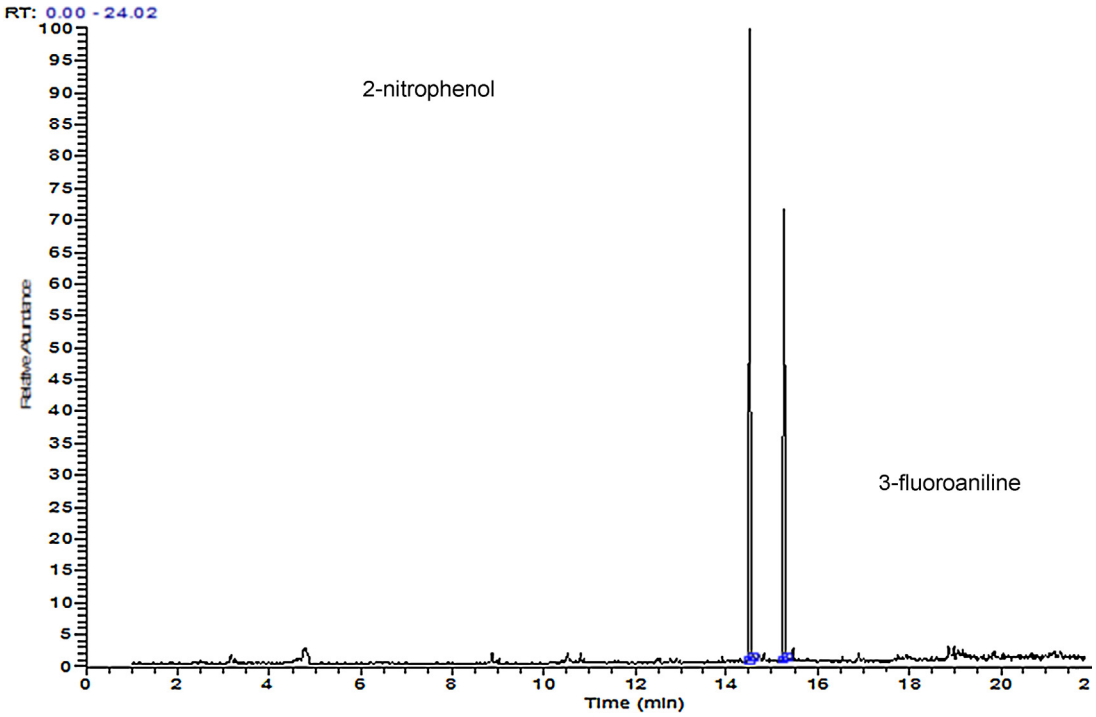

Fig. 2. VOC profile liberated by *L. monocytogenes* NCTC 11994 with 2-nitrophenyl-β-D-glucoside and 2-[(3-fluorophenyl) carbamoylamino]acetic acid.

required for the generation of 2-nitrophenol and 3-fluoroaniline, respectively, after overnight incubation (Table 2). A time study, carried out in duplicate using *L. monocytogenes* NCTC 11994 at an initial inoculum of 1–1.5 × 10^7 CFU ml^{-1} milk, indicated that 2-nitrophenol was liberated by both *L. monocytogenes* samples in whole milk after 7 h incubation and 3-fluoroaniline after 9 h incubation. Table 3 outlines liberated VOCs over the 21 h time period.

Six additional milk types were used to investigate milk type on VOCs liberated. Table 4 outlines the VOCs generated by *L. monocytogenes* NCTC 11994 and uninoculated milk samples in seven milk types. 2-Nitrophenol and 3-fluoroaniline were generated by *L. monocytogenes* when inoculated in all seven milk types. VOCs were not detected in any blank milk sample, with the exception of one unpasteurised milk sample which liberated 10.1 μg ml^{-1} of 2-nitrophenol. This sample did not produce 3-fluoroaniline. The remaining two blank unpasteurised milk samples did not generate either VOC.

Fifteen Gram-positive bacteria, including a second strain of *L. monocytogenes*, and 2 Gram-negative bacteria were tested in triplicate with the method. Table 5 outlines VOCs liberated by all species. Out of the six species of *Listeria* tested, four liberated both 2-nitrophenol and 3-fluoroaniline. *L. grayi* did not produce either VOC. *L. seeligeri* produced 3-fluoroaniline but only generated 2-nitrophenol in trace amounts that were below the LOQ, therefore *L. seeligeri* was considered to be negative for 2-nitrophenol production. *E. faecalis*, *E. faecium* and *L. acidophilus* liberated both 2-nitrophenol and 3-fluoroaniline. Other species tested did not produce 2-nitrophenol or 3-fluoroaniline. Growth of all other species was inhibited in LEB with added selective supplement.

Both 2-nitrophenol and 3-fluoroaniline were generated by *L. monocytogenes* as a result of β-glucosidase and hippuricase activity, respectively, indicating that they were potentially useful markers for *L. monocytogenes* contaminated milk. The potential for a rapid method of *L. monocytogenes* identification was demonstrated by the time of 9 h that was required to generate both VOCs. However, the initial inoculum used here was high at 1–1.5 × 10^7 CFU ml^{-1} of whole milk; this would be significantly higher than would be found in contaminated food samples. Since *L. monocytogenes* liberated both 2-nitrophenol and 3-fluoroaniline in 100% of inoculated milk samples, any sample where VOCs are not produced can be considered negative for *L. monocytogenes*. However, method sensitivity data indicated that this only applies at initial inocula of at least 1–1.5 × 10^2 CFU ml^{-1} after overnight incubation. Approved culture-based methods for the detection of *L. monocytogenes* are highly sensitive; the limit of detection of the ISO 11290 method is 5–100 CFU per 25 g of food [18,19]. However, enrichment procedures are necessary prior to isolation via culturing to allow growth of *L. monocytogenes* to levels sufficient to enable detection; 5–6 days may be required to obtain results [5].

Table 2
Sensitivity of VOC method.

Initial inoculum (CFU ml^{-1} milk)	2-Nitrophenol concentration (μg ml^{-1})	3-Fluoroaniline concentration (μg ml^{-1})
1–1.5 × 10^7	3.0	1.2
1–1.5 × 10^6	6.4	1.7
1–1.5 × 10^5	9.9	1.8
1–1.5 × 10^4	13.2	1.1
1–1.5 × 10^3	25.7	0.4
1–1.5 × 10^2	5.8	0.3
1–1.5 × 10^1	1.7	ND
1–1.5 × 10^0	ND	ND

ND: not detected.

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Table 3
VOCs liberated by *L. monocytogenes* NCTC 11994 over time.

Time (h)	Sample 1		Sample 2	
	2-Nitrophenol conc. ($\mu g\,ml^{-1}$)	3-Fluoroaniline conc. ($\mu g\,ml^{-1}$)	2-Nitrophenol conc. ($\mu g\,ml^{-1}$)	3-Fluoroaniline conc. ($\mu g\,ml^{-1}$)
1	ND	ND	ND	ND
3	ND	ND	ND	ND
5	ND	ND	ND	ND
7	0.6	ND	0.6	ND
9	13.4	0.3	13.3	0.3
11	14.8	1.9	14.6	1.9
13	10.3	2.9	10.3	3.0
15	6.3	2.8	6.6	3.1
17	4.6	3.2	4.8	3.5
19	3.2	3.3	3.5	3.6
21	2.4	3.5	2.3	3.6

ND: not detected.

Table 4
Effect of milk type and VOCs liberated by *L. monocytogenes* NCTC 11994 and uninoculated milk.

Sample	Milk type	Mean 2-nitrophenol concentration ±1 standard deviation[a] ($\mu g\,ml^{-1}$)	Mean 3-fluoroaniline concentration ±1 standard deviation[a] ($\mu g\,ml^{-1}$)
LM	Whole	1.3 ± 0.1	4.0 ± 0.1
Blank	Whole	ND	ND
LM	Semi-skimmed	1.1 ± 0.5	7.2 ± 0.4
Blank	Semi-skimmed	ND	ND
LM	Skimmed	6.2 ± 0.7	8.6 ± 0.7
Blank	Skimmed	ND	ND
LM	Unpasteurised	36.6 ± 1.6	9.2 ± 0.9
Blank	Unpasteurised*	ND	ND
Blank	Unpasteurised*	10.1	ND
Blank	Unpasteurised*	ND	ND
LM	Unhomogenised	2.8 ± 0.7	6.0 ± 0.8
Blank	Unhomogenised	ND	ND
LM	Whole goats	2.5 ± 0.3	5.6 ± 0.2
Blank	Whole goats	ND	ND
LM	Soya	1.4 ± 0.9	12.1 ± 0.5
Blank	Soya	ND	ND

Blank = unpasteurised samples shown separately; LM = *Listeria monocytogenes* NCTC 11994; Blank = uninoculated milk; ND: not detected.
[a] $n = 3$.

Table 5
VOCs generated by other species of bacteria inoculated in whole milk.

Bacteria	Growth in LEB*	Mean 2-nitrophenol concentration ±1 standard deviation[a] ($\mu g\,ml^{-1}$)	Mean 3-fluoroaniline concentration ±1 standard deviation[a] ($\mu g\,ml^{-1}$)
Gram-positive bacteria			
L. monocytogenes NCTC 10537	+	0.9 ± 0.8	9.8 ± 0.7
L. welshimeri	+	1.1 ± 0.04	2.1 ± 0.07
L. innocua	+	0.5 ± 0.05	9.8 ± 0.09
L. seeligeri	+	<LOQ	0.5 ± 0.05
L. ivanovii	+	89.5 ± 6.5	0.7 ± 0.07
L. grayi	+	ND	ND
E. faecalis	–	10.0 ± 2.4	2.0 ± 0.1
E. faecium	+	39.5 ± 1.28	0.6 ± 0.06
L. acidophilus	+	39.2 ± 12.2	3.8 ± 2.1
L. lactis	–	ND	ND
C. xerosis	–	ND	ND
C. striatum	–	ND	ND
B. lichenformis	–	ND	ND
B. cereus	–	ND	ND
B. subtilis	–	ND	ND
Gram-negative bacteria			
S. enteritidis	–	ND	ND
E. coli NCTC 12079	–	ND	ND

+ = growth; – = growth inhibited; ND = not detected (< LOD); <LOQ = less than LOQ therefore VOC not quantifiable.
[a] $n = 3$.

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Immunoassays, such as enzyme-linked immunosorbent assays (ELISA), have been developed for rapid detection of *L. monocytogenes*. These methods target antibodies specific to *Listeria*, and although less time-consuming than classical culturing, they are less sensitive with detection limits of 10^4–10^6 CFU ml^{-1} [20]. Detection of *L. monocytogenes* in artificially contaminated milk samples using MALDI-TOF MS was recently reported [21]. Here, two enrichment steps were required and detection of *L. monocytogenes* in milk spiked with an initial inoculum of 1 CFU ml^{-1} was possible after 30 h. The detection of VOCs liberated from enzyme substrates could potentially be used as a rapid method for the detection of *L. monocytogenes.* However, currently the method has several limitations; these include use of a small sample size and a limited range of reference bacteria. Testing the method with a larger sample size, in addition to examining the VOCs generated by other strains of all *Listeria* species would be necessary. In addition, samples used to test the method were artificially contaminated; further investigations would be required using real-life samples.

L. monocytogenes liberated VOCs when inoculated in the seven milk types tested; VOCs were not produced by the uninoculated milk samples with the exception of one unpasteurised milk sample. The selective agents incorporated into the culture medium would not inhibit every organism present in untreated unpasteurised milk. In order to suppress the remaining uninhibited species and hence eliminate VOC signals generated by background flora, an examination of the combination of selective agents could be considered. However, both VOCs are generated by *L. monocytogenes*, therefore the liberation of 2-nitrophenol without 3-fluoroaniline would not indicate *L. monocytogenes* contamination. Consequently, the unpasteurised milk sample liberating 2-nitrophenol would be classified as negative for *L. monocytogenes* contamination.

The majority of *Listeria* species liberated both VOCs. *L. grayi* and *L. seeligeri* did not liberate VOCs in quantifiable amounts therefore VOCs were designated as not detected. *L. seeligeri* and *L. grayi* may require a longer incubation time for the liberation of sufficient VOCs for quantification purposes, particularly since *Listeria* species are known to be slow growing [5]. The VOC profiles of all VOC generating *Listeria* species are displayed in Fig. 3, which particularly illustrates the overlapping of the VOC profiles of *L. monocytogenes* NCTC 10357 and *L. innocua*. Although differentiation of *L. monocytogenes* from all of the other *Listeria* species was not possible, the detection of other *Listeria* species can be an advantage. In addition, the different clustering for the VOC profiles for both *Listeria monocytogenes* species could be useful to investigate differences in metabolism between members of the same species. The presence of non-pathogenic *Listeria* species can often be an indicator of inadequate standards of hygiene during food production and therefore the non-pathogenic species are often regarded as a marker for *L. monocytogenes* [22].

Three other species tested (*E. faecalis*, *E. faecium* and *L. acidophilus*) also liberated 2-nitrophenol and 3-fluoroaniline. Due to the liberation of VOCs by these species and by other *Listeria* species, it would not be possible to determine whether VOCs generated in an unknown milk sample indicates *L. monocytogenes* contaminated milk. The inhibition of false positive VOC signals would require further investigations into alternative combinations

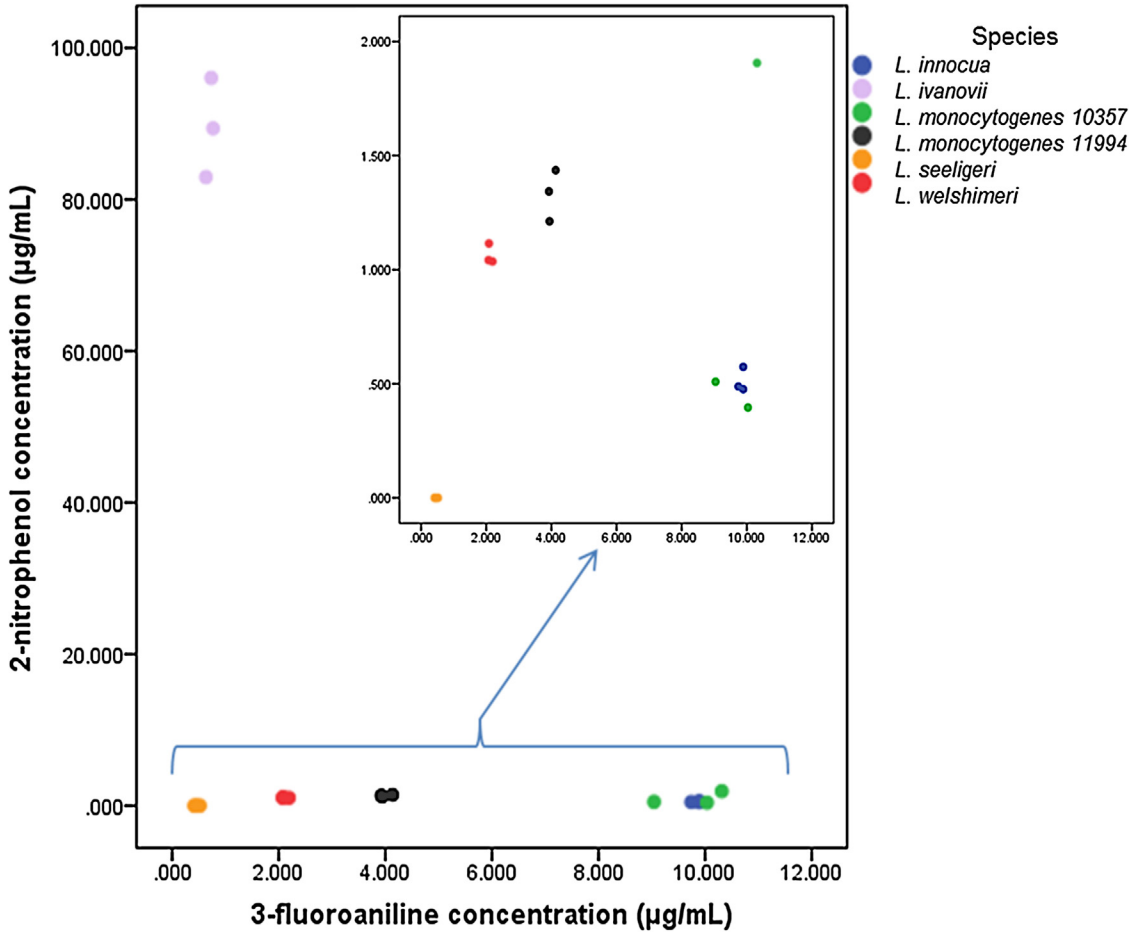

Fig. 3. VOCs liberated by all *Listeria* species (including both *L. monocytogenes* NCTC 10357 and *L. monocytogenes* NCTC 11994).

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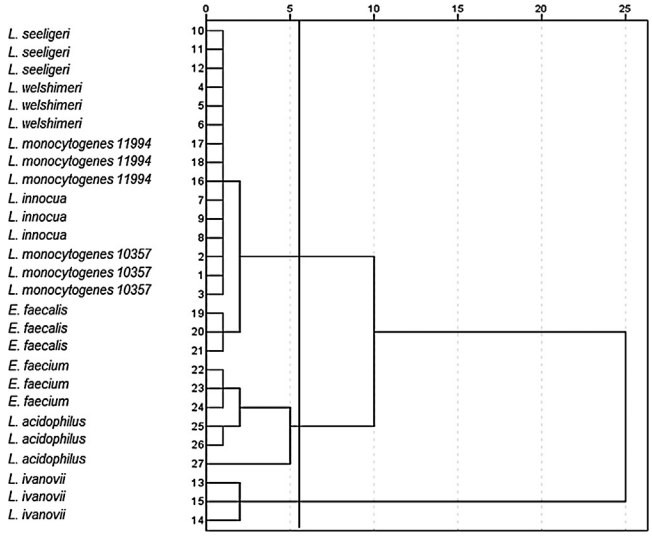

Fig. 4. Cluster analysis on the VOC profiles of all VOC liberating species.

of selective agents to suppress the growth of VOC generating species. All other species tested did not produce 2-nitrophenol or 3-fluoroaniline. Growth of all other species was inhibited in LEB with added selective supplement. The susceptibility or resistance of tested species to the selective agents is supported in literature [23–25].

In order to ascertain whether *L. monocytogenes* could be differentiated from all other species based on VOCs liberated, VOC profiles were subject to cluster analysis with groupings made from similarities between VOC profiles. Each repeat was treated as a separate sample ($n = 3$ for all species). Species that did not generate any VOCs were excluded from analysis. Three clusters formed (Fig. 4) with one cluster composed of both strains of *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *E. faecalis*. This demonstrated that there was no clear distinction between the VOC profiles for these species. *L. ivanovii* clustered separately. The remaining cluster consisted of *E. faecium* and *L. acidiophilus*. The larger amounts of 2-nitrophenol liberated could be attributed to separate clustering of *L. ivanovii*, *E. faecium* and *L. acidiophilus*. Cluster analysis indicated that detection of *L. monocytogenes* contaminated milk using quantitative differences in VOCs liberated would not be possible using the current method. Identifying the source of milk contamination by the presence or absence of VOCs is likely to be more successful; particularly as the amount of *L. monocytogenes* present in contaminated food samples is likely to vary considerably thereby affecting the amount of VOCs liberated.

4. Conclusion

These investigations demonstrate that the VOC method could potentially be used to detect *L. monocytogenes* contamination in milk. The limit of detection was $1–1.5 \times 10^2$ CFU ml^{-1} after overnight incubation. Absence of VOCs could indicate milk free from *L. monocytogenes* contamination, although this applies only under conditions tested here. Further investigations would be required, for example by testing longer incubation times with smaller initial inoculum sizes, to determine whether absence of VOCs indicates the presence of *L. monocytogenes*. The enrichment broth with added selective agents cycloheximide, nalidixic acid and acriflavine HCl was included for the suppression of background flora and for the inhibition of species closely related to *L. monocytogenes*. The method was not entirely specific for *L. monocytogenes* therefore in order to develop the method further investigations into other selective agent combinations would be required. The new method could potentially be applied to other food types and used as a rapid method of identifying the source of *L. monocytogenes* in investigations of outbreaks and hence eliminating non-contaminated food samples as causes of suspected food-borne illness.

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References

[1] B.C. Swaminathan, P. Gerner-Smidt, The epidemiology of human listeriosis, Microbes Infect. 9 (2007) 1236–1243.
[2] M. Zunabovic, K.J. Domig, W. Kneifel, Practical relevance of methodologies for detecting and tracing of *Listeria monocytogenes* in ready-to-eat foods and manufacture environments–a review, LWT-Food Sci. Technol. 44 (2011) 351–362.
[3] E.C.D. Todd, S. Notermans, Surveillance of listeriosis and its causative pathogen, *Listeria monocytogenes*, Food Control 22 (2011) 1484–1490.
[4] J. McLaughlin, The relationship between *Listeria* and listeriosis, Food Control 7 (1996) 187–193.
[5] U. Gasanov, D. Hughes, P.M. Hansbro, Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review, FEMS Microbiol. Rev. 29 (2005) 851–875.
[6] S. Ellis, B.S. Walker, The action of hippuricase on ring substituted derivates of hippuric acid, J. Biol. Chem. 142 (1942) 291–297.
[7] J.D. Perry, L. Archer, A.L. Jones, A.L. James, M. Ford, F.K. Gould, Detection of carboxypeptidases as taxonomic markers for gram-negative bacteria, Lett. Appl. Microbiol. 26 (1998) 359–362.
[8] S.B. Feresu, D. Jones, Taxonomic studies on *Brochothrix, Erysipelothrix Listeria* and atypical *lactobacilli*, J. Gen. Microbiol. 134 (1988) 1165–1183.
[9] A.P. Snyder, D.B. Shoff, G.A. Eiceman, D.A. Blyth, J.A. Parsons, Detection of bacteria by ion mobility spectrometry, Anal. Chem. 63 (1991) 526–529.
[10] A.P. Snyder, M. Miller, D.B. Shoff, G.A. Eiceman, D.A. Blyth, J.A. Parsons, Enzyme-substrate assay for the qualitative detection of microorganisms by ion mobility spectrometry, J. Microbiol. Methods 14 (1991) 21–32.
[11] N.J.C. Strachan, F.J. Nicholson, I.D. Ogden, An automated sampling system using ion mobility spectrometry for the rapid detection of bacteria, Anal. Chim. Acta 313 (1995) 63–67.
[12] L.-H. Guillemot, M. Vrignaud, P.R. Marcoux, C. Rivron, T.-H. Tran-Thi, Facile and fast detection of bacteria via the detection of exogenous volatile metabolites released by enzymatic hydrolysis, Phys. Chem. Chem. Phys. 15 (2013) 15840–15844.
[13] Y.W. Chu, B.Y. Wang, D.A. Engebretson, J.R. Carey, Single step, rapid identification of pathogenic microorganisms in a culture bottle, Analyst 138 (2013) 5879–5885.
[14] C.L. Lonsdale, B. Taba, N. Queralto, R.A. Lukaszewski, R.A. Martino, P.A. Rhodes, S.H. Lim, The use of colorimetric sensor arrays to discriminate between pathogenic bacteria, PLoS One 8 (2013) e62726, doi:http://dx.doi.org/10.1371/journal.pone.0062726.
[15] J.R. Carey, K.S. Suslick, K.I. Hulkower, J.A. Imlay, K.R.C. Imlay, C.K. Ingison, J.B. Ponder, A. Sen, A.E. Wittrig, Rapid identification of bacteria with a disposable colorimetric sensing array, J. Am. Chem. Soc. 133 (2011) 7571–7576.
[16] M. Safavieh, M.U. Ahmed, E. Sokullu, A. Ng, L. Braescu, M. Zourob, A simple cassette as point-of-care diagnostic device for naked-eye colorimetric bacteria detection, Analyst 139 (2014) 482–487.
[17] E. Tait, J.D. Perry, S.P. Stanforth, J.R. Dean, Identification of volatile organic compounds produced by bacteria using HS-SPME-GC–MS, J. Chromatogr. Sci. 52 (2014) 363–373.
[18] Anonymous, Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection and Enumeration of *Listeria monocytogenes*: Part 1: Detection Method, Geneva: International Organization for Standardization (ISO), 1996 ISO 11290-1, Amendment in 2004.
[19] Churchill, H. Lee, J.C. Hall, Detection of *Listeria monocytogenes* and the toxin listeriolysin O in food, J. Microbiol. Methods 64 (2006) 141–170.
[20] A. Roda, M. Mirasoli, B. Roda, F. Bonvicini, C. Colliva, P. Reschiglian, Recent developments in rapid multiplexed bioanalytical methods for foodborne pathogenic bacteria detection, Microchim. Acta 178 (2012) 7–28.
[21] S. Jadhav, D. Sevior, M. Bhave, E.A. Palombo, Detection of *Listeria monocytogenes* from selective enrichment broth using MALDI–TOF mass spectrometry, J. Proteomics 97 (2014) 100–106.

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[22] R.J. Gilbert, J. de Louvois, T. Donovan, C. Little, K. Nye, C.D. Ribeiro, J. Richards, D. Roberts, F.J. Bolton, Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale, Commun. Dis. Public Health 3 (2000) 163–167.
[23] R.R. Beumer, W.C. Hazeleger, *Listeria monocytogenes*: diagnostic problems, FEMS Immunol. Med. Microbiol. 35 (2003) 191–197.
[24] C.N. Jacobsen, The influence of commonly used selective agents on the growth of *Listeria monocytogenes*, Int. J. Food Microbiol. 50 (1999) 221–226.
[25] S. Mathur, R. Singh, Antibiotic resistance in food lactic acid bacteria–a review, Int. J. Food Microbiol. 105 (2005) 281–295.

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